DEVELOPMENT AND EVALUATION OF AN *ISOSPORA SUIS* SPOROZOITE NEUTRALIZING ANTIBODY ASSAY IN SWINE TESTICULAR CELLS

A Thesis Presented to

The College of Arts and Sciences

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

---

by

Ann M. Steger

September 1997

DEVELOPMENT AND EVALUATION OF AN ISOSPORA SUIS SPOROZOITE NEUTRALIZING ANTIBODY ASSAY IN SWINE TESTICULAR CELLS

by

Ann M. Steger

Approved by Committee

Rodney A. Rogers, PhD

Richard Wacha, PhD

Douglas P. Quick, PhD

DEVELOPMENT AND EVALUATION OF AN ISOSPORA SUIS SPOROZOITE NEUTRALIZING ANTIBODY ASSAY IN SWINE TESTICULAR CELLS

An abstract of a Thesis by

Ann M. Steger

September 1997

Drake University

An *in vitro* Isospora suis sporozoite neutralizing antibody assay has been developed in swine testicular (ST) cells. This assay measures the titer of neutralizing antibodies present in sera, milk and monoclonal antibody culture fluids by determining both the amount of intracellular sporozoites by immunofluorescence and the cytopathic effect in ST cells. A monoclonal antibody against the apical complex of I. suis sporozoites inhibited the infection of sporozoites *in vitro*. Hyperimmune sera produced in goats and guinea pigs inhibited sporozoite infection in ST cells. Milk samples from gilts vaccinated with I. suis contained neutralizing antibodies, however the titers did not correlate with protection observed in nursing pigs after challenge.

TABLE OF CONTENTS

INTRODUCTION AND REVIEW OF LITERATURE

Isospora suis was first described by Biester and Murray in 1934 and was isolated from pigs in Iowa (Biester & Murray, 1934). In 1978, the organism was recognized to be of major clinical and economic importance when it was identified as the pathogen that caused neonatal porcine coccidiosis (Stuart et al., 1978). I. suis, a coccidial parasite, is an obligate intracellular protozoan that causes disease in nursing pigs that are less than two weeks of age. The genus Isospora is assigned to the phylum Apicomplexa and family Eimeridiidae. There are nine different species of coccidia that are found in swine in the United States (Lindsay et al., 1984a) but Isospora suis has been the only one shown to cause neonatal coccidiosis (Stuart et al., 1978; Tubbs, 1986). The other eight coccidia are species of Eimeria which have not been shown to cause disease in pigs (Lindsay et al., 1984a).

Isospora suis is distibuted worldwide and has been associated with porcine coccidiosis in the United States (Stuart & Lindsay, 1986; Lindsay, 1989; Tubbs & Dekich, 1986; Fitzgerald et al., 1988; Johnson et al., 1992; Bergeland, 1981; Marti & Hale, 1986; Jarvinen, 1990; Vetterling, 1966), Canada (Robinson & Morin, 1982; Lindsay, 1990), Sweden (Nilsson & Martinsson, 1984), Brazil (Sayd & Kawazoe, 1996), Australia (Driesen et al., 1993), Germany (Otten, 1995) and the Netherlands (Eysker et al., 1994). Since 1980, I. suis has been isolated from 11-22% of preweaning diarrhea cases (Stuart & Lindsay, 1986; Lindsay, 1989; Fitzgerald et al., 1988; Johnson et al., 1992; Burkhardt, 1995; Lindsay, 1990); 11-18% of postweaning diarrhea cases (Fitzgerald et al., 1988; Johnson et al., 1992; Burkhardt, 1995); and 12-18% of all porcine diarrhea cases (Bergeland, 1981; South Dakota ADRDL, 1989-95) submitted to United States diagnostic laboratories. Fecal samples collected from herds throughout the United States have

indicated a 30 to 90% prevalence of I. suis (Tubbs & Dekich, 1986; Jarvinen, 1990).

I. suis causes non-hemorrhagic diarrhea in nursing pigs that occurs primarily between five and fourteen days of age (Lindsay, 1989; Tubbs, 1986). Generally, diarrhea develops approximately 4 to 6 days post-infection (PI) and continues for a duration of 5 or 6 days (Robinson et al., 1983; Tubbs, 1986). Pigs infected with this parasite have a high morbidity rate but a low to moderate mortality rate (Lindsay et al., 1985; Stuart et al., 1978).

The morbidity caused by an I. suis infection results in decreased growth rates and unthriftiness in pigs. This, in turn, causes high economic losses to swine producers (Waddell, 1993; Dudley, 1990). The additional time and feed it takes for an I. suis infected pig to reach market weight depends on the extent of the infection and damage to the intestinal mucosa (Stuart, 1982). It has been indicated that pigs with diarrhea for 4 days have average weight gains of 0.11 pound per day less than their healthy littermates from birth to market (Waddell, 1993). It is this additional time and feed necessary for an I. suis infected pig to reach market weight that causes economic losses to the swine industry.

The life cycle of Isospora suis is complex and consists of three distinct phases - sporogony, excystation and endogeny. Studies of various aspects of these three life cycle phases have been reported by Lindsay, et al. (Lindsay et al., 1980; Lindsay et al., 1982; Lindsay et al., 1983c). Sporogony occurs outside the host while excystation and endogeny occur inside the host. The exact mechanisms of these life cycle phases are not completely understood (Dubremetz, 1993).

Sporogony.

Sporogony, or the exogenous phase, is the part "of the life cycle that occurs outside the host in the environment in which the unsporulated, noninfectious oocyst develops into the sporulated, infectious oocyst" (Lindsay & Todd, 1993). Temperature, moisture and

oxygen conditions must be optimal for sporulation to occur. Optimal sporulation occurs between 20°C and 37°C and is inhibited at temperatures less than 20°C and greater than 37°C (Lindsay et al., 1982; Lindsay, 1990). Sporogony is an aerobic process that requires adequate oxygen for respiration (Lindsay, 1990; Lindsay & Todd, 1993). Unsporulated I. suis oocysts, measuring approximately 21.2 X 17.9 mm, are passed in the feces and contain a uninucleate sporont with a peripherally located nucleus (Lindsay et al., 1982). One to two "hazy bodies" are located in unsporulated oocysts between the sporont and the oocyst wall (Lindsay et al., 1980; Lindsay et al., 1982; Tubbs, 1986). These translucent "hazy bodies" are a diagnostic characteristic of unsporulated I. suis oocysts in fecal samples. At the beginning of sporogony, a nuclear division occurs in which the sporont divides into two uninucleate sporoblasts. Each sporoblast then undergoes a nuclear division that results in the formation of a sporocyst. A final nuclear division results in the production of four sporozoites in each sporocyst (Lindsay et al., 1982; Lindsay, 1990). Thus, a total of three nuclear divisions occur throughout the sporogony phase of the life cycle.

Excystation.

Excystation is the phase of the life cycle that occurs in a newly inoculated host following the ingestion of sporulated, infectious oocysts. Excystation is a two-step process consisting of the alteration of the oocyst wall followed by the alteration of the sporocyst wall. The oocyst wall is altered *in vivo* by hydrochloric acid in the stomach. It is altered *in vitro* by sodium hypochlorite (Lindsay et al., 1983c). The alteration of the oocyst wall facilitates permeablility of bile salts and pancreatic enzymes (Lindsay & Todd, 1993), especially trypsin, which activates the sporozoites (Lindsay, 1990; Lindsay & Todd, 1993). Activated sporozoites enter the intestinal lumen when pancreatic enzymes cause gaps in the sporocyst and oocyst walls.

In vitro excystation studies using chemical and mechanical disruption of the oocyst wall and enzyme treatment have been reported by Lindsay, et al. (1983c). Before the sporozoites excyst from the sporocyst, they become slightly swollen and exhibit "intermittent tumbling movements" inside the sporocyst when activated by bile and trypsin (Lindsay et al., 1983c). Shortly after sporozoite movement is initiated, the sporocyst wall displays indentations and then separates, releasing the sporozoites. Sporozoites just released from sporocysts are short and broad but sporozoites that have been excysted for 5 to 10 minutes are elongate and highly motile. Elongate sporozoites, on the average, measure 11.7 X 3.8 mm (Lindsay et al., 1983c). Sporozoite movements involve "rapid forward gliding, side-to-side flexion and a probing action with the anterior end" (Lindsay et al., 1983c; Lindsay, 1984b). The anterior end of the sporozoite is pointed whereas the posterior end is rounded.

Endogeny.

The endogenous phase of the I. suis life cycle consists of two main developmental stages, asexual and sexual. These stages develop intracellularly in villus epithelial cells (Sangster et al., 1978; Stuart et al., 1978), mainly in the jejunum and ileum (Lindsay 1990; Sangster, Seibold, & Mitchell) of the small intestine of swine. Intracellular stages can be observed in parasitiphorous vacuoles in the distal third of villus epithelial cells, basal to the host cell nucleus (Lindsay et al., 1980; Lindsay et al., 1992). An excysted sporozoite penetrates an intestinal epithelial cell by invagination of the host cell plasma membrane which forms the parasitiphorous vacuole (Lindsay et al., 1980; Lindsay & Todd, 1993). In severe I. suis infections, in addition to the ileum and jejenum, endogenous stages can be identified in crypt epithelial cells (Lindsay et al., 1980; Stuart et al., 1978), the cecum and the colon (Harleman & Meyer, 1985; Lindsay, 1990).

Asexual Development.

Endogenous stages are first observed at approximately 36 hours PI as uninucleate zoites (immature meronts) in mucosal smears of the jejunum and ileum (Lindsay et al., 1980). Mature binucleate type I meronts can be seen in tissue sections and mucosal smears 3 days PI and average 14.4 X 7.8 mm (Lindsay et al., 1980; Lindsay et al., 1983b). Type I meronts undergo division by endodyogeny and after approximately 24 hours produce two elongate type I daughter merozoites (Lindsay et al., 1983b; Lindsay, 1990). These, in turn, may then reproduce "by binary fission or endodyogeny in a variable number of consecutive divisions in the same host cell" and produce a total of 2 to 14 type I merozoites (Lindsay et al., 1980). Type I merozoites are crescent-shaped, are located side-by-side in a single parasitiphorous vacuole and measure 15.2 X 6.5 mm in mucosal smears. Type I endogenous stages are most prolific at 4 days PI, at which time they are observed in increased numbers (Lindsay et al., 1980; Robinson et al., 1983). Type I merozoites leave the host cell and subsequently penetrate adjacent epithelial cells. At 4 days PI, type II multinucleate meronts are observed that contain three to twelve nuclei and average 15.4 X 7.5 mm in mucosal smears. Type II merozoites are produced from type II meronts by endodyogeny. Type II merozoites in each parasitiphorous vacuole are smaller than type I merozoites and range from two to sixteen in number with average dimensions of 9.7 X 3.4 mm (Lindsay et al., 1980). Type II merozoites develop into the sexual stages of I. suis (Lindsay et al., 1983a; Tubbs, 1986).

Lindsay et al. (1983b) investigated the motility of meronts and merozoites and determined that motile meronts have a "sharply pointed anterior end". Meronts, like sporozoites, exhibit gliding and flexing movement lasting about 3 to 10 seconds. Meronts also probe with their anterior end, which is similar to sporozoite probing action (Lindsay et al., 1983b; Lindsay, 1984b). Meronts penetrate the villus epithelial cells of the host and when they leave their particular host cell, they penetrate adjacent host cells.

Sexual Development.

No sexual stages are seen until approximately 4 days PI (Lindsay et al., 1980; Lindsay et al., 1992). By 5 days PI, both mature and immature sexual stages are identifiable (Lindsay et al., 1980). Sexual stages of I. suis consist of two types of gamonts, microgamonts and macrogamonts. Microgametes are produced within microgamonts via monocentric microgametogenesis. This process consists of biflagellate, sperm-like, multinucleate microgametes budding off "the surface of a single spherical residual body" (Lindsay et al., 1992a; Lindsay & Todd, 1993). The actively motile microgametes arrange themselves around the residual body in the parasitiphorous vacuole and eventually leave this vacuole in order to locate and fertilize a macrogamont (Lindsay et al., 1980; Lindsay & Todd, 1993). Mature macrogamonts are elongate to ovoid and measure 9.4 X 6.5 mm in tissue sections (Lindsay et al., 1980). A macrogamete is fertilized by a microgamete to form an intracellular zygote (Lindsay & Todd, 1993). This zygote develops a cell wall to form an oocyst (Lindsay et al., 1992a; Tubbs, 1986) which in tissue sections measures 14.9 X 12.1 mm and in mucosal smears 18.4 x 16.1 mm (Lindsay et al., 1980). Oocysts contain a granular sporont and can be distinguished from macrogamonts in tissue sections by their distinct oocyst wall (Lindsay et al., 1980). These oocysts can be seen in feces as early as four (Robinson et al., 1983) to five (Lindsay et al., 1980; Lindsay, 1990) days post-inoculation.

In vitro.

Isolated Isospora suis sporozoites have been inoculated into tissue culture and the endogenous stages observed. Sporozoites could be seen penetrating and producing progeny by endodyogeny. All of these cultures failed to produce any sexual stages of I. suis (Fayer et al., 1984; Lindsay & Blagburn, 1987). Fayer et al. (1984) inoculated I. suis sporozoites into the following cell lines: Mardin-Darby bovine kidney (MDBK) cells,

embryonic bovine trachea (EBTr) cells, bovine colon (BC) cells, and porcine kidney (PK) cells. Within 60 minutes at 37°C the sporozoites penetrated the EBTr and MDBK cells. At 24 hours PI, intracellular parasites were observed in all four cultures. Intracellular sporozoites were "located adjacent to the host cell nucleus and were surrounded by a parasitiphorous vacuole" (Fayer et al., 1984). By 48 hours PI, endodyogeny had occurred in 62-66% of the intracellular sporozoites resulting in two second generation progeny. These progeny resembled the sporozoite in appearance. However, the intracellular parasites appeared to have decreased in number by 5 days PI (Fayer et al., 1984).

Lindsay and Blagburn (1987) have also completed *in vitro* studies with I. suis using primary cell cultures, specifically primary porcine kidney (PPK) cells and primary fetal bovine kidney (PFBK) cells. These cultures were tested for I. suis propagation because primary cultures from the host organism tend to better support the development of parasites. The sporozoites penetrated the cells of both cultures and developed into motile type I meronts and merozoites. However, asexual development progressed until multinucleate, type II meronts were observed in only the PPK cell culture.

The sporozoites penetrated the PPK and PFBK cells and were located adjacent to the host cell nucleus (Lindsay & Blagburn, 1987). The location of the sporozoites in this study were the same as that described by Fayer et al. (1984). In some cases, multiple sporozoites infected a single host cell which was observed in both cell types at 24 hours post inoculation. The average dimensions of the sporozoites in both cultures measured 9.0 x 3.4 mm. By 36 hours PI, endodyogeny had occurred in both cell lines and the progeny consisted of type I binucleate meronts and paired type I merozoites. "A few motile type I merozoites were observed in the culture medium" (Lindsay & Blagburn, 1987). Endodyogenous development continued through 72 hours PI, producing numerous type I meronts and type I merozoites which were both intracellular and motile in the culture medium. Occasionally these motile type I meronts and merozoites were "observed

penetrating or leaving cultured cells. No adverse effects were observed on host cells due to entrance or exit of type I meronts or merozoites" (Lindsay & Blagburn, 1987). Considering the *in vivo* pathology of I. suis infections, a question arises as to why the entrance or exit of these asexual stages did not cause cell lysis. Perhaps cell death did not appear to occur immediately and was overlooked. Another possibility is that the culture media, containing 2% fetal bovine serum (FBS), contributed enough nutrients for the cells to repair the damage created by the parasite.

In the PFBK cultures, type I meronts and merozoites were the only developmental stages seen through 144 hours PI. In the PPK cell cultures, beginning at 96 hours PI, multinucleate type II meronts were observed, containing four to ten nuclei and measuring 18.1 x 12.1 mm. However, no type II merozoites were produced by these meronts and they were never observed to be motile in the culture medium and were therefore considered to be non-viable. Through 144 hours PI, type I meronts and merozoites were observed both intracellular and motile in the culture media (Lindsay & Blagburn, 1987).

Lindsay and Blagburn (1987) have identified motile, type I asexual stages of Isospora suis in tissue culture. They also describe motile type I and type II meronts derived from the mucosal surface of infected pigs (Lindsay et al., 1983c). The *in vitro* results described correlate with the *in vivo* results, except that the type II meronts propagated *in vitro* were not motile. The *in vitro* results described above are also supported by the findings of Lindsay et al. (1980) that multiple infections of single swine epithelial cells exist *in vivo*.

Investigators at Ambico, Inc., Dallas Center, Iowa have successfully completed the I. suis life cycle, from sporozoite to oocyst, in swine testicular (ST) cells (In press, 1993). The sporozoites attach and penetrate the ST cells and are located in a parasitiphorous vacuole adjacent to the nucleus of the infected cell. By 2 days post-inoculation (PI), type I merozoites are observed as two crescent-shaped organisms situated side-by-side within a

parasitiphorous vacuole. Beginning at 2 days PI, intracellular merozoites escape into the culture medium, leaving the previously infected cell destroyed. Merozoites are visible "swimming" free in the culture medium. These "free-swimming" merozoites subsequently infect another ST cell and continue their development. Three days PI, type II intracellular merozoites are visible within a single parasitiphorous vacuole. The number of intracellular type II merozoites ranges from 4 to 20 per parasitiphorous vacuole and have the appearance of "bunches of bananas". Type II merozoites are readily found free in the culture medium 4 to 7 days PI and can be differentiated from type I merozoites due to their smaller size. The sexual stages are observed within host cells as early as 6 days PI and intracellular oocysts are visible from 7 to 12 days PI.

Isospora suis sporozoites have been inoculated into various types of cultured cells in which the sporozoites penetrated and produced progeny through asexual (Fayer et al., 1984; Lindsay et al., 1987) and sexual development (In press, 1993). These results demonstrate that I. suis sporozoites are infectious in various cell lines.

There are many published reports which show that genus-specific antibodies in colostrum and serum inhibit the infection of Apicomplexan parasites *in vitro*. Colostral antibodies from cows previously infected with Cryptosporidium parvum inhibit the penetration of C. parvum sporozoites in cultured cells (Doyle et al., 1993). Similarily, colostrum from cows immunized with Eimeria acervulina antigens significantly reduces the number of E. acervulina sporozoites observed in cultured cells (Fayer & Jenkins, 1992). Long and Rose (1972) reported that "treatment of [chick kidney cell] cultures with immune globulin appeared to inhibit sporozoite invasion". Immune serum antibodies have been shown to decrease the invasion and growth of various Apicomplexan species *in vitro*. Convalescent serum from BALB/c mice previously infected with Plasmodium yoelii reduces the invasion rate and growth of P. falciparum *in vitro* (Ray et al., 1994). Guo et al. (1984) reported that P. fragile growth in culture was inhibited upon incubation with

post-vaccination serum from rhesus monkeys. Mouse serum antibodies against Eimeria tenella inhibit homologous sporozoite infectivity *in vitro* (Crane et al., 1986).

Other published reports indicate that monoclonal antibodies inhibit the infection of Apicomplexan parasites *in vitro*. Monoclonal antibodies to the various life cycle stages of Apicomplexan parasites have been isolated and analyzed for their neutralizing characteristics *in vitro*. Monoclonal antibodies have been prepared against Toxoplasma gondii surface proteins (Grimwood & Smith, 1996) and the Eimeria acervulina apical complex (Sasai et al., 1996) that inhibit invasion of tachyzoites and sporozoites, respectively, in cultured cells. A monoclonal antibody of Eimeria adenoeides inhibits the invasion of cultured cells with E. adenoeides and E. tenella sporozoites (Augustine, 1991). Incubation of Eimeria bovis sporozoites with a surface protein monoclonal antibody significantly reduces penetration of MDBK cultured cells (Whitmire et al., 1988).

Reports of the propagation of Isospora suis in cultured cells and the neutralization of Apicomplexan parasites in the presence of colostral, serum or monoclonal antibodies suggested the feasibility of developing an *in vitro* sporozoite neutralizing antibody assay being developed for I. suis. The purpose of this thesis investigation was to develop an assay that detects Isospora suis sporozoite neutralizing antibodies in serum, milk and monoclonal antibody culture fluids. This assay will measure the level of neutralizing antibodies and will benefit both pharmaceutical companies involved in the development of Isospora suis vaccines and diagnostic laboratories in monitoring outbreaks and collecting epidemiology information. This technology can also be applied to other Apicomplexan parasites for vaccine development and as a diagnostic test for the detection of exposure to these parasites.

MATERIALS AND METHODS

Production of oocyst seeds.

Isospora suis oocysts were produced in specific pathogen-free (SPF) neonatal pigs, breed line PC C22, from H & K Enterprise in Nevada, Iowa. Pigs were allowed to nurse the sow for 3-5 days before being brought to Ambico, Inc. At that time they were placed in isolation boxes and subcutaneously administered 3.0 ml of Clostridium perfringens Type C & D Antitoxin (Clostrax-BCD™, Grand Laboratories, Inc., Larchwood, IA) and 1.0 ml intramuscularly of 100 mg Iron Dextran Complex (Iron Hydrogenated Dextran, Western Veterinary Supply, Inc., Porterville, CA). Pigs were fed twice daily with Littermilk® medicated pig milk replacer (Land O'Lakes, Inc., Fort Dodge, IA) containing oxytetracycline and neomycin base antibiotics and additionally supplemented with liquid Spectam® Scour-Halt™ spectinomycin antibiotic (Rhone Merieux, Inc., Athens, GA). Each pig was inoculated between 5 and 12 days of age with 6,000 to 18,000 I. suis oocysts per dose, depending on the age of the pig. Six to nine days post-inoculation, the pigs were sacrificed and both the cecum and large intestine were collected. The intestinal contents were released by first unwinding and longitudinally cutting the cecum and large intestine then rinsing the intestinal tissue with phosphate-buffered saline (PBS, Addendum 1). In order to break up the feces, the intestinal contents were briefly blended on pulse cycle with a Hamilton Beach® 7 Speed Blendmaster™ blender. The oocysts present in the intestinal contents were pelleted by centrifugation at 1,900 x g at 4°C for 30 minutes. The oocyst pellets were re-suspended in 2.5% potassium dichromate (w/v) in distilled water and sporulated by aeration for 24 to 72 hours at 25°C (Lindsay et al., 1982). The aeration procedure included attaching a plastic 10 ml pipet to the end of a Second Nature Whisper® 800 fish-tank aerator (Willinger Brothers, Inc., Oakland, NJ), placing the pipet in the oocyst-dichromate seed, and gently "bubbling" air through the seed. The titer of sporulated

oocysts was determined by counting them on a hemacytometer and the oocyst seeds were stored at 4°C for subsequent use.

Purification of oocysts.

Oocysts were purified using a modification of the procedure described by Lindsay et al. (1980, 1982). Oocyst seeds were mixed with 70 - 100% Sheather's sugar solution (Addendum 1) to a final concentration between 10% and 12.5% (v/v) and were centrifuged at 1,900 x g for 15 minutes at room temperature (21-23°C). Oocysts were removed from the meniscus using a 1.4 cm diameter wire loop and were washed in phosphate-buffered saline (PBS, Addendum 1) by centrifuging at 2,080 x g for 30 minutes at 4°C. After washing, the oocysts were re-suspended in Earle's salts minimum essential medium with non-essential amino acids (EMNE, Addendum 1) containing 800 mg/ml gentamicin.

Isolation of sporozoites.

Purified oocysts were homogenized at room temperature using a Wheaton™ Overhead Stirrer (cat. no. 903475) that lysed the three layers of the oocyst wall. Homogenization time differed between purified oocyst seeds and was dependent upon the oocyst titer and the remaining contaminating fecal debris. Homogenization was continued until approximately 50 to 70 percent of the oocysts were lysed, as determined by microscopic evaluation. If the oocysts were homogenized beyond 50 to 70 percent lysis, some of the sporocysts became lysed, subsequently destroying the sporozoites. The homogenate was centrifuged at 1,900 x g for 10 to 15 minutes at 4°C and the pellet, containing the sporocysts, was re-suspended with a 5 to 10X volume of 0.75% bile/0.25% trypsin (v/v) mixture (Lindsay et al., 1983a). The sporocysts were incubated in the bile/trypsin mixture in a 37°C incubator for 30 minutes with gentle agitation (60-90 rpm) to enzymatically release the sporozoites. The bile/trypsin mixture was washed from the

sporozoites by centrifuging at 1,900 x g for 10 to 15 minutes at 4°C. The sporozoite pellet was resuspended in the test medium (see "Media" section below) to a specified concentration of sporozoites per milliliter (see "Inoculum" section below). The media and sporozoite inoculum levels varied as the parameters of the neutralization assay were being developed.

Growth and maintenance of ST cells.

A diploid, swine testicular (ST) cell line (cell passages 90 to 100) was used for neutralization assay development. ST cells were planted in Earle's salts minimum essential medium with non-essential amino acids (EMNE, Addendum 1) containing 10% bovine calf serum, defined (HyClone Laboratories, Inc., Logan, Utah). Ninety-six-well microtiter plates were planted with 2 x 10^4 ST cells per well. ST cells were grown to confluency in air containing 3-5% CO_2 and were maintained with EMNE containing 2% bovine calf serum.

Neutralization assay.

Test samples (serum, milk and monoclonal antibody fluids) were diluted in the desired medium (see "Media" section below) and an equal volume containing a constant number of sporozoites was added to each test sample dilution. The sporozoites were neutralized by the test samples at 22°C or 37°C in glass tubes sealed with a rubber stopper. ST cells were rinsed with medium, inoculated with neutralized sporozoites, and adsorbed at 37°C for 60 to 90 minutes. Following adsorption, the ST cells were rinsed with basal medium and incubated at 37°C in a 3-5% CO_2 atmosphere. Cells were stained either with Diff-Quik (Baxter) differential stain or by indirect immunofluorescent assay.

Staining Procedures.

Differential Stain.

Prior to staining with Diff-Quik differential stain, ST cells were incubated at 37°C in 3-5% CO_2 for 6 to 8 days following sporozoite adsorption. The ST cells were fixed and stained with the reagents supplied in the Diff-Quik differential staining kit. The stained ST cells were observed for cytopathic effects (CPE) indicating sporozoite infection. If no cell layer remained, the sporozoite infection caused CPE and destroyed the entire cell monolayer. However, if the cell monolayer remained intact (i.e., the cells stained purple), then the antibodies present in the test sample neutralized the sporozoites, preventing infection.

Indirect immunofluorescent assay (IFA).

Following adsorption of the sporozoites to the ST cells, the cells were rinsed with EMNE and were incubated at 37°C in 3-5% CO_2 for 1 hour. The ST cells were subsequently fixed with ice cold 100% methanol for 1 hour at -20°C. The intracellular sporozoites were probed with the supernatant fluid from pass 10 of the monoclonal antibody 2G3-H7, concentrated 10-fold with a Diaflo Ultrafilter, 50,000 molecular weight cut-off (Amicon, Inc., Beverly, MA). A 1:50 dilution of the 10X concentrated supernatant fluid from the monoclonal antibody 2G3-H7 was incubated with the cell monolayers in each well for 30 minutes in a humidified chamber at 37°C with 3-5% CO_2. Excess antibody was removed by washing 3 times with PBS (pH 7.2). Fluorescein-labeled goat anti-mouse IgG conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MA) was incubated with the cells for 30 minutes in a humidified chamber at 37°C with 3-5% CO_2. The cells were rinsed of excess conjugate by washing 3 times with PBS. After the final wash, 50% glycerol:PBS (v/v) was added to the ST cell monolayers and the assay was read using an inverted, fluorescent compound microscope. Sporozoite infection was evident by fluorescence of intracellular sporozoites in the ST cell monolayer. ST cell

monolayers that were virtually void of fluorescence indicated that sporozoite infection had been prevented and that antibodies in the test sample had neutralized the sporozoites.

Parameters examined for development of the neutralization assay.

Media.

Media tested included Earle's salts minimum essential medium with non-essential amino acids (EMNE, Addendum 1). The supplements tested were 0.01 mg/ml DEAE Dextran and 0.02 mg/ml trypsin.

Inoculum.

Inoculation volume and number of sporozoites were tested in 96 well flat-bottom microtiter plates. Inoculation parameters per well were 0.2 ml, 0.1 ml or 0.05 ml each containing 5,000, 10,000 or 20,000 sporozoites.

Neutralization time and temperature.

Neutralization time was tested by incubation of I. suis sporozoites with diluted test samples for 15, 30, 60, and 90 minutes at temperatures of 21°C and 37°C.

Adsorption to ST cells.

Medium containing a low concentration of bovine calf serum (BCS) was tested to determine if its presence would stabilize the sporozoites and lengthen their infection time post-excystation. The following media were tested: EMNE alone and EMNE containing 2, 5, 10 or 20% BCS; DMEM alone and DMEM containing 2, 5 or 10% BCS.

Atmospheric conditions.

The propagation of I. suis in ST cells was tested in the presence and absence of 3-5% CO_2.

Immune serum antibodies.

Production of guinea pig hyperimmune serum to oocyst lysate.

Three 350-400 gram guinea pigs (Sasco, Omaha, NE) were immunized 3 times at 3 week intervals with an oocyst lysate antigen obtained from purified sporulated oocysts treated with glass beads or a bile/trypsin solution which released the sporozoite antigens from the oocysts and sporocysts (Lindsay et al., 1983a). The first and second subcutaneous vaccinations each contained 0.27 mg of oocyst lysate (BCA Protein Assay, Pierce, Rockford, IL) in Freund's Complete Adjuvant (Sigma Chemical Company, St. Louis, MO), and the final aqueous intramuscular vaccination contained 0.27 mg of oocyst lysate protein.

Ten days after final vaccination, guinea pigs were terminally anesthetized with 20mg/lb Ketamine HCl (Keteset®, Fort Dodge Laboratories, Inc., Fort Dodge, IA) and 2.3 mg/lb Xylazine (Rompun®, Bayer, Shawnee Mission, KS) and then bled via heart puncture. Serum was separated from the clotted blood by centrifuging at 1,900 x g at 4°C for 15 to 30 minutes followed by heating for 30 minutes at 56°C to inactivate complement. Sera were stored at -20°C until ready for use.

Production of goat hyperimmune serum to sporozoites.

A 75 pound, one year old female goat (Al Buseman, Dumont, IA) was immunized 4 times at 3 week intervals with a sporozoite lysate antigen. The sporozoites were isolated from purified, sporulated oocysts excysted with a bile/trypsin solution (Lindsay, et al., 1983a). The first subcutaneous vaccination contained 0.065 mg of sporozoite antigen in Freund's Complete Adjuvant and was followed by the second and third vaccinations

containing 0.125 mg and 0.250 mg sporozoite protein, respectively, in Freund's Incomplete Adjuvant (Sigma Chemical Company, St. Louis, MO). A final aqueous intramuscular vaccination was administered containing 0.50 mg sporozoite protein.

Five days after the final vaccination, the goat was stunned and exsanguinated. Serum was separated from the clotted blood by centrifuging at 1,900 x g at 4°C for 15 to 30 minutes followed by heating for 30 minutes at 56°C to inactivate complement. Serum was stored at -20°C until ready for use.

Production of negative pig serum.

Negative pig serum was obtained from Cesarean-derived, colostrum deprived pigs. One to four hours after birth, the pigs were stunned and exsanguinated. Serum was separated from the clotted blood by centrifuging at 1,900 x g at 4°C for 15 to 30 minutes followed by heating for 30 minutes at 56°C to inactivate complement. Sera were stored at -20°C until ready for use.

Production of test milk samples.

Experiment 1.

Gilts #830, 789, 119 and 118 were vaccinated three times by feeding them a corn-milk mash containing 200,000, 400,000 and 800,000 I. suis sporulated oocysts at 5, 3 and 1 week pre-farrow, respectively. Gilts #121 and 122 were orally vaccinated once with 200,000 sporulated oocysts at 5 weeks pre-farrow, followed by 4 intramuscular vaccinations with a freeze-thawed lysate of 3×10^6, 4×10^6, 4.5×10^6 oocysts in Freund's Incomplete Adjuvant at 4, 3, and 2 weeks pre-farrow, respectively. Finally, at 1 week pre-farrow they were vaccinated with an aqueous preparation containing 4.5×10^6 lysed oocysts. Gilts #82 and 123 were non-vaccinated controls. Milk samples were obtained from all gilts 5 days post-farrow. Milk samples were clarified by

centrifuging at 1,900 x g at 4°C for 30 minutes and the middle layer of milk was heat inactivated for 30 minutes at 56°C. Milk samples were stored at -20°C until ready for use.

Nursing pigs were challenged orally with 25,000 I. suis sporulated oocysts at 5 days of age. Pigs were observed daily for signs of diarrhea and death. A necropsy was performed on any pigs that died post-challenge to determine the cause of death. The morbidity incidence, (number of pigs with diarrhea / total pigs in the litter) x 100, and percent mortality, (number of pigs that died from I. suis / total pigs in the litter) x 100, were calculated for each litter. Group morbidity and mortality means were calculated.

Experiment 2.

Gilts #180, 182 and 183 were vaccinated three times intramuscularly with a freeze-thawed lysate of 2 x 10^6 oocysts in Freund's Incomplete Adjuvant at 5 and 3 weeks pre-farrow and an aqueous vaccination with 2 x 10^6 lysed oocysts 1 week pre-farrow. Gilts #193 and 194 were vaccinated once orally with 200,000 sporulated oocysts at 6 weeks pre-farrow, followed by the same intramuscular vaccinations seen in gilts #180, 182 and 183. Gilts #176, 177, 178 and 179 were non-vaccinated controls. Milk samples were obtained from all gilts 3 days post-farrow. Milk samples were clarified by centrifuging at 1,900 x g at 4°C for 30 minutes and the middle layer of milk was heat inactivated for 30 minutes at 56°C. Milk samples were stored at -20°C until ready for use.

Nursing pigs were challenged orally with 50,000 I. suis sporulated oocysts at 3 days of age. Pigs were observed daily for signs of diarrhea and death. Pigs that died post-challenge were necropsied to determine the cause of death. The morbidity incidence and percent mortality were calculated for each litter and group as in

Experiment 1.

Production of monoclonal antibody fluids.

An oocyst/sporozoite lysate, containing 0.62 mg/ml of protein, was prepared by homogenizing 1.8 x 10^6 freeze-thawed, purified oocysts with a Wheaton™ Overhead Stirrer. This lysate was sent to the Iowa State University Cell and Hybridoma Facility in Ames, Iowa for the preparation of I. suis sporozoite hybridomas. After screening the primary hybridomas against oocyst lysate proteins by immunoblot, two hybridomas (2G3 and 1E2) were chosen for further expansion and cloning. The secondary hybridomas of 2G3 were screened for antibody production to oocyst lysate by Western blot and ELISA. Clones 2G3-H7 and 2G3-C4 were chosen for expansion followed by propagation and passage in DMEM + 10% horse serum (Irvine Scientific, Santa Ana, CA). The supernatant fluids from the hybridoma cultures were stored at -20°C for future analysis on ELISA and the serum neutralization assay.

A portion of the 2G3-H7 monoclonal antibody supernatant fluid from pass 10 was concentrated 10-fold using a Diaflo Ultrafilter with a 50,000 molecular weight cut-off membrane (Amicon, Inc., Beverly, MA).

Enzyme-linked immunosorbant assay (ELISA).

An enzyme-linked immunosorbant assay (ELISA) was used to screen test samples (serum, milk, monoclonal fluids) for the presence of antibodies to oocyst/sporozoite lysate. Oocyst/sporozoite lysate antigen (prepared from purified, homogenized, freeze-thawed oocysts) was added to Immulon-2 U-bottom, 96-well microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) at 21 ng/well. Plates were sealed and incubated overnight at 4°C. The wells were washed 3 times with PBS + 0.05% Tween-20 (PBST) and the unbound sites were blocked with 10% fetal bovine serum (JRH Biosciences,

Lenexa, KS) in PBST (v/v) for 2 hours at 22°C. The blocking solution was aspirated and diluted test samples (serum, milk, monoclonal fluids) were added to each well and the plates were incubated at 22°C for 1 hour. Wells were washed of unbound antibody with PBST 3 times and species-specific, peroxidase-labeled conjugate (Kirkegaard & Perry Laboratories or Bethyl Laboratories) was added to each well. Plates were incubated for 1 hour at 22°C. Wells were washed 4 times with PBST and ABTS, a 1 component peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MA), was added to all wells. Plates were sealed and incubated at 22°C for 30 minutes. The optical density of each well was determined at 405 nm using an SLT 340 ATTC plate reader (SLT Labinstruments, Austria, Europe). An $OD_{405} \geq 0.200$ (after the average blank OD_{405} had been subtracted) was considered positive for antibody to oocyst/sporozoite lysate. The titer of each antibody sample was determined as the reciprocal of the highest dilution having an $OD_{405} \geq 0.200$.

RESULTS

An *in vitro* assay in swine testicular cells has been developed that detects Isospora suis sporozoite neutralizing antibodies in serum, milk and monoclonal antibody culture fluids. Described below are the results obtained for the neutralization assay and the neutralization titers obtained from test samples which include hyperimmune sera and post-vaccination sow milk samples.

Examination of parameters for the development of the antibody neutralization assay.

Media.

Initial adsorption and penetration of sporozoites to ST cells was not affected by the addition of trypsin or DEAE dextran to the EMNE medium. Therefore, EMNE medium without trypsin or DEAE dextran was determined to be the optimal basal medium used throughout the neutralization assay.

Inoculum and Adsorption.

All assays described in this section were performed in 96-well microtiter plates containing confluent monolayers of ST cells and were incubated at 37°C for 7 days post-inoculation. Inoculated monolayers were stained with Diff-Quik (Baxter) differential stain and observed for cytopathic effect (CPE) which directly correlates to the level of sporozoite infection of the monolayer (i.e., the higher the percent CPE, the greater the sporozoite infection).

Table 1 shows the percent CPE of ST cell monolayers that were inoculated with different levels of sporozoites in varying volumes of EMNE alone or supplemented with bovine calf serum. ST cell monolayers that were inoculated with 2×10^4 I. suis

sporozoites in 0.2 ml, 0.1 ml or 0.05 ml EMNE containing 2%, 5%, 10% or 20% BCS resulted in 90 to 100% cytopathic effect (CPE) of the entire cell monolayers. ST cells inoculated with 2 x 10^4 sporozoites in 0.2 ml and 0.05 ml EMNE alone resulted in 40% and 100% CPE, respectively. Eighty to 95% CPE was observed in ST cell monolayers inoculated with 1 x 10^4 sporozoites in 0.05 ml EMNE containing 2%, 5%, 10% or 20% BCS and in 0.1 ml EMNE containing 20% BCS. ST cells inoculated with 1 x 10^4 sporozoites in 0.1 ml EMNE containing 2%, 5%, or 10% BCS resulted in approximately 60 to 75% CPE of the cell monolayers. Less than 50% CPE was observed in monolayers inoculated in EMNE containing 2%, 5% or 10% BCS with 1 x 10^4 sporozoites in 0.2 ml and 5 x 10^3 sporozoites in 0.2 ml, 0.1 ml and 0.05 ml. ST cells inoculated in EMNE containing 20% BCS with 1 x 10^4 sporozoites in 0.2 ml and 5 x 10^3 sporozoites in 0.1 ml and 0.05 ml resulted in 70 to 75% CPE. Only 10% CPE was observed in ST cell monolayers inoculated with 5 x 10^3 sporozoites in 0.2 ml EMNE containing 20% BCS.

Table 2 shows the percent CPE of ST cell monolayers that were inoculated with different levels of sporozoites in varying volumes of Dulbecco's Modified Eagle's Medium (DMEM) alone or supplemented with bovine calf serum. ST cell monolayers that were inoculated with 2 x 10^4 L suis sporozoites in 0.1 ml or 0.05 ml DMEM containing 2%, 5% or 10% bovine calf serum (BCS) resulted in 100% CPE. However, ST cells inoculated in DMEM alone with 2 x 10^4 sporozoites in 0.1 ml or 0.05 ml resulted in 99% and 95% CPE, respectively. ST cells inoculated with 1 x 10^4 sporozoites in 0.1 ml or 0.05 ml DMEM containing 2%, 5% or 10% BCS resulted in 80 to 95% CPE of the monolayers. ST cell monolayers inoculated with 1 x 10^4 sporozoites in 0.1 ml or 0.05 ml DMEM alone or 5 x 10^3 sporozoites in DMEM containing 2%, 5%, or 10% BCS resulted in 10 to 40% CPE, while ST cells inoculated with 5 x 10^3 sporozoites in 0.1 ml and 0.05 ml DMEM alone did not cause CPE of the cell monolayer.

Table 1. Percent cytopathic effect of ST cell monolayers vs. inoculation of I. suis sporozoites in EMNE medium.

Test No.	Basal Medium	Percent BCS[1]	Inoculum		Percent CPE[2]
			No. of Sporozoites	Volume (ml)	
1	EMNE[3]	0	2×10^4	0.2 ml	40%
		2%			98%
		5%			95%
		10%			90%
		20%			95%
2	EMNE	2%	2×10^4	0.1 ml	100%
		5%			100%
		10%			100%
		20%			98%
3	EMNE	0	2×10^4	0.05 ml	100%
		2%			100%
		5%			100%
		10%			100%
		20%			98%
4	EMNE	2%	1×10^4	0.2 ml	40%
		5%			40%
		10%			40%
		20%			70%
5	EMNE	2%	1×10^4	0.1 ml	75%
		5%			60%
		10%			60%
		20%			90%
6	EMNE	2%	1×10^4	0.05 ml	80%
		5%			80%
		10%			95%
		20%			95%
7	EMNE	2%	5×10^3	0.2 ml	5%
		5%			5%
		10%			5%
		20%			10%
8	EMNE	2%	5×10^3	0.1 ml	30%
		5%			20%
		10%			20%
		20%			75%
9	EMNE	2%	5×10^3	0.05 ml	20%
		5%			20%
		10%			50%
		20%			75%

1. BCS: Bovine calf serum.
2. CPE: Cytopathic effect of the ST cell monolayer.
3. EMNE: Earle's salts minimum essential medium with non-essential amino acids medium.

Table 2. Percent cytopathic effect of ST cell monolayers vs. inoculation of I. suis sporozoites in DMEM medium.

Test No.	Basal Medium	Percent BCS[1]	Inoculum		Percent CPE[2]
			No. of Sporozoites	Volume (ml)	
1	DMEM[3]	0	2×10^4	0.1 ml	99%
		2%			100%
		5%			100%
		10%			100%
2	DMEM	0	2×10^4	0.05 ml	95%
		2%			100%
		5%			100%
		10%			100%
3	DMEM	0	1×10^4	0.1 ml	10%
		2%			90%
		5%			90%
		10%			80%
4	DMEM	0	1×10^4	0.05 ml	20%
		2%			90%
		5%			95%
		10%			90%
5	DMEM	0	5×10^3	0.1 ml	0
		2%			20%
		5%			40%
		10%			15%
6	DMEM	0	5×10^3	0.05 ml	0
		2%			30%
		5%			30%
		10%			20%

1. BCS: Bovine calf serum.
2. CPE: Cytopathic effect of the ST cell monolayer.
3. DMEM: Dulbecco's Modified Eagle's Medium.

The results described above provided evidence for increased adsorption of sporozoites to ST cells at lower inoculum volumes. For the I. suis sporozoite neutralization assay it was determined that 1.0 to 2.0 x 10^4 I. suis sporozoites would be the optimal inoculation per well of confluent ST cell monolayers using a 0.05 ml inoculum volume in EMNE containing 2 to 5% bovine calf serum.

Neutralization Time and Temperature.

I. suis sporozoites are fully neutralized, as indicated by the absence of CPE, following a 60 minute incubation with antibody samples (pre-diluted 1:8) at either 37°C or 21°C (Table 3). However, sporozoite penetration of ST cells decreases by 90 minutes, therefore a 60 minute neutralization at 37°C was used in the I. suis sporozoite neutralization assay.

Table 3. The effect of incubation time and temperature of sporozoites with antibody and their neutralization in ST cells.

Sample	Neutralization Time	Temperature	Percent CPE[1]	Neutralization[2]
negative serum[3] (pre-vaccination)	15 min	21°C	100%	negative
	30		100%	negative
	60		100%	negative
	90		90%	negative
positive serum[4] (hyperimmune)	15 min	21°C	50%	negative
	30		20%	negative
	60		0	POSITIVE
	90		0	POSITIVE
sporozoite control[5]	15 min	21°C	100%	N.D.[6]
	30		100%	N.D.
	60		95%	N.D.
	90		25%	N.D.
negative serum (pre-vaccination)	15 min	37°C	95%	negative
	30		95%	negative
	60		95%	negative
	90		85%	negative
positive serum (hyperimmune)	15 min	37°C	70%	negative
	30		85%	negative
	60		10%	POSITIVE
	90		0	POSITIVE
sporozoite control	15 min	37°C	75%	N.D.
	30		100%	N.D.
	60		100%	N.D.
	90		65%	N.D.

1. CPE: Cytopathic effect of the ST cell monolayer.
2. Neutralization: as determined by the percent CPE caused by sporozoites in ST cells. Negative neutralization = CPE > 20%; Positive neutralization = CPE $\leq$ 10%.
3. Negative serum: guinea pig pre-vaccination serum, pre-diluted 1:8 with sporozoites.
4. Positive serum: guinea pig hyperimmune serum, pre-diluted 1:8 with sporozoites.
5. Sporozoite control: 2×10^4 sporozoites per well in 5% bovine serum.
6. N.D.: Not determined; neutralization cannot be determined in sporozoite control sample.

Atmospheric Conditions.

There was no difference in the propagation of I. suis sporozoites in ST cells in the presence or absence of 3-5% CO_2.

Staining Procedures.

Neutralization assays that were fixed 1 hour post-inoculation, stained and read by immunofluorescence gave comparable results to neutralization assays that were fixed 7 days post-inoculation, stained with Diff-Quik differential stain and read by cytopathic effect.

Inhibition of sporozoite penetration of ST cells by immune serum antibodies.

Pre-incubation of I. suis sporozoites for 60 minutes with hyperimmune sera generated in guinea pigs and goats completely eliminated infection of ST cells at dilutions of 1:16 and 1:32, respectively, while the pre-vaccination sera from both species did not prevent infection of the sporozoites in ST cells (Table 4). The guinea pig and goat hyperimmune sera IgG antibody titers against oocyst/sporozoite lysate antigen on an ELISA were determined to be 5,120 and 2,560, respectively, while the pre-vaccination sera of both species gave no reaction on the ELISA, resulting in a titer $\leq$20 (Table 4).

Table 4. Guinea pig and goat serum neutralizing antibody titers vs I. suis sporozoites and IgG antibody titers vs I. suis oocyst/sporozoite lysate antigen on ELISA.

Serum Sample	Neutralizing Antibody Titer[1] vs Sporozoties	IgG Antibody Titer[2] vs Oocyst/Sporozoite Lysate
guinea pig pre-vaccination	$\leq$2	$\leq$20
guinea pig hyperimmune	16	5,120
goat pre-vaccination	$\leq$2	$\leq$20
goat hyperimmune	32	2,560

1. Neutralizing Antibody Titer: The inverse of the serum dilution at which I. suis sporozoite penetration is inhibited in swine testicular cells.
2. IgG Antibody Titer: The inverse of the serum dilution that gives an optical density value $\geq$0.200 at 405 nm against oocyst/sporozoite lysate antigen on an ELISA.

Inhibition of sporozoite penetration of ST cells by monoclonal antibody fluid.

Pre-incubation of I. suis sporozoites for 60 minutes with the 2G3-H7 monoclonal antibody culture fluid, generated at pass 10, did not completely inhibit the infection of sporozoites in ST cells, while the same fluid concentrated 10-fold (v/v) completely eliminated the infection of the sporozoites in ST cells at a 1:4 dilution (Table 5). The unconcentrated and 10X concentrated 2G3-H7 monoclonal antibody culture fluids from pass 10 had IgG ELISA antibody titers against oocyst/sporozoite lysate antigen of 400 and 3,200, respectively (Table 5).

Table 5. 2G3-H7 monoclonal antibody culture fluid neutralizing antibody titers vs I. suis sporozoites and IgG antibody titers vs I. suis oocyst/sporozoite lysate antigen on ELISA.

Monoclonal Antibody	Neutralizing Antibody Titer[1] vs Sporozoties	IgG Antibody Titer[2] vs Oocyst/Sporozoite Lysate
2G3-H7 culture fluid[3]	≤2	400
2G3-H7 culture fluid, 10X conc[4]	4	3,200

1. Neutralizing Antibody Titer: The inverse of the monoclonal culture fluid dilution at which I. suis sporozoite penetration is inhibited in swine testicular cells.
2. IgG Antibody Titer: The inverse of the monoclonal culture fluid dilution that gives an optical density value ≥0.200 at 405 nm against oocyst/sporozoite lysate antigen on an ELISA.
3. Monoclonal antibody 2G3-H7 culture fluid from *in vitro* pass 10.
4. Monoclonal antibody 2G3-H7 culture fluid *in vitro* pass 10 concentrated 10-fold (v/v).

Effect of sow vaccination in the production of neutralizing antibodies in milk.

Experiment 1.

The sporozoite neutralizing antibody titers and the IgG and IgA ELISA antibody titers in the 5 day post-farrow milk samples from gilts in Experiment 1 are shown in Table 6. The four gilts in group A vaccinated orally with three doses of I. suis oocysts had neutralizing antibody titers in their 5 day post-farrow milk ranging from <2 to 48, with a

geometric mean titer (GMT) of 18.75. Neither gilt #121 nor #122, vaccinated orally once with oocysts and intramuscular three times with sporozoite lysate, produced neutralizing antibodies in their milk 5 days post-farrow. The non-vaccinated gilts of group C, #82 and #123, had sporozoite neutralizing antibody titers in their 5-day post-farrow milk samples of 3 and <2, respectively, with a GMT of 1.5.

Table 6. Experiment 1 sow neutralizing antibody titers vs I. suis sporozoites and IgG and IgA antibody titers vs I. suis oocyst/sporozoite lysate antigen on ELISA.

Gilt No.	Inoculation Group	Neutralizing Antibody Titer[1]	ELISA Antibody Titer[2] IgG(γ)	IgA(α)
118	A[3]	3	8	32
119		≤2	8	128
789		24	8	128
830		48	8	32
GMT[6]		**18.75**	**8.0**	**80**
121	B[4]	≤2	128	32
122		≤2	32	32
GMT		**<2**	**80**	**32**
82	C[5]	3	≤2	32
123		≤2	2	2
GMT		**1.5**	**1.0**	**17**

1. Neutralizing Antibody Titer: The inverse of the milk dilution at which I. suis sporozoite penetration is inhibited in swine testicular cells.
2. ELISA Antibody Titer: The inverse of the milk dilution that gives an optical density value ≥0.200 at 405 nm against oocyst/sporozoite lysate antigen on an ELISA.
3. Group A Vaccination: 200,000, 400,000 and 800,000 I. suis oocysts orally at 5, 3 and 1 week pre-farrow, respectively.
4. Group B Vaccination: 200,000 I. suis oocysts orally at 5 weeks pre-farrow followed by intramuscular vaccinations with oocyst/sporozoite lysate at 4, 3, 2 and 1 week pre-farrow.
5. Group C Vaccination: Non-vaccinated controls.
6. GMT: Geometric mean titer; determined by calculating the statistical mean of the treatment group.

The post-challenge morbidity incidence and mortality data for each litter is given in Table 7. The litters suckling the gilts of group A had morbidity incidences ranging from 22 to 100% with a group mean of 80.5% and litter mortalities ranging from 0 to 18% with a

mean of 7.75%. Pigs suckling the gilts from group B had 100% morbidity and no mortality post-challenge. The litters suckling the non-vaccinated gilts of group C had morbidity incidences of 90 and 100% with a 95% mean and mortalities of 40 and 100% with a group mean of 70%.

Table 7. Experiment 1 post-challenge morbidity incidence and percent mortality of litters suckling vaccinated gilts.

Gilt No.	Inoculation Group	Litter Number	Morbidity Incidence[1]	Percent Mortality[2]
118	A[3]	5	100%	0
119		4	22%	0
789		2	100%	18%
830		1	100%	13%
Group Mean[6]			**80.5%**	**7.75%**
121	B[4]	6	100%	0
122		3	100%	0
Group Mean			**100%**	**0**
82	C[5]	9	90%	40%
123		7	100%	100%
Group Mean			**95%**	**70%**

1. Morbidity Incidence = (number of pigs with diarrhea / total pigs in litter) x 100.
2. Percent Mortality = (number of pigs that died from I. suis challenge / total number of pigs in litter) x 100.
3. Group A Gilt Vaccination: 200,000, 400,000 and 800,000 I. suis oocysts orally at 5, 3 and 1 week pre-farrow, respectively.
4. Group B Gilt Vaccination: 200,000 I. suis oocysts orally at 5 weeks pre-farrow followed by intramuscular vaccinations with oocyst/sporozoite lysate at 4, 3, 2 and 1 week pre-farrow.
5. Group C Gilt Vaccination: Non-vaccinated controls.
6. Group Mean: The statistical mean of each treatment group.

Experiment 2.

The sporozoite neutralizing antibody titers and the IgG and IgA ELISA antibody titers in the 3 day post-farrow milk samples from gilts in Experiment 2 are shown in Table 8. The three gilts in group A vaccinated three times intramuscular with sporozoite lysate

had neutralizing antibody titers in their 3 day post-farrow milk ranging from <2 to 12, with a mean titer of 5.0. Group B gilts #193 and #194, vaccinated orally once with oocysts and intramuscular three times with sporozoite lysate, had sporozoite neutralizing antibody titers in their 3-day post-farrow milk samples of 3 and <2, respectively, with a GMT of 1.5. The four non-vaccinated control gilts of group C had neutralizing antibody titers ranging from <2 to 3 in their 3 day post-farrow milk, with a mean titer of 2.25.

Table 8. Experiment 2 sow neutralizing antibody titers vs I. suis sporozoites and IgG and IgA antibody titers vs I. suis oocyst/sporozoite lysate antigen on ELISA.

Animal No.	Inoculation Group	Neutralizing Antibody Titer[1]	ELISA Antibody Titer[2] IgG(γ)	IgA(α)
180	A[3]	3	32	512
182		12	32	32
183		≤2	32	80
GMT[6]		**5.0**	**32**	**208**
193	B[4]	3	32	32
194		≤2	8	128
GMT		**1.5**	**20**	**80**
176	C[5]	≤2	≤2	32
177		3	≤2	32
178		3	8	128
179		3	2	128
GMT		**2.25**	**2.5**	**80**

1. Neutralizing Antibody Titer: The inverse of the milk dilution at which I. suis sporozoite penetration is inhibited in swine testicular cells.
2. ELISA Antibody Titer: The inverse of the milk dilution that gives an optical density value ≥0.200 at 405 nm against oocyst/sporozoite lysate antigen on an ELISA.
3. Group A Vaccination: 3 intramuscular vaccinations at 5, 3 and 1 week pre-farrow with oocyst/sporozoite lysate.
4. Group B Vaccination: 200,000 I. suis oocysts orally at 6 weeks pre-farrow followed by intramuscular vaccinations with oocyst/sporozoite lysate at 5, 3 and 1 week pre-farrow.
5. Group C Vaccination: Non-vaccinated controls.
6. GMT: Geometric mean titer; determined by calculating the statistical mean of the treatment group.

The post-challenge morbidity incidence and mortality data for each litter is given in Table 9. The litters suckling the gilts of group A had morbidity incidences ranging from 50 to 100% with a group mean of 83.3% and litter mortalities ranging from 25 to 57% with a mean of 40.7%. Pigs suckling the gilts from group B had 100% morbidity and 29% mortality after challenge. The litters suckling the non-vaccinated gilts of group C had 100% morbidity and mortalities of 38 to 70% with a group mean of 56%.

Table 9. Experiment 2 post-challenge morbidity incidence and percent mortality of litters suckling vaccinated gilts.

Gilt No.	Inoculation Group	Litter Number	Morbidity Incidence[1]	Percent Mortality[2]
180	A[3]	5	50%	25%
182		6	100%	57%
183		7	100%	40%
Group Mean[6]			**83.3%**	**40.7%**
193	B[4]	12	100%	29%
194		13	100%	29%
Group Mean			**100%**	**29%**
176	C[5]	2	100%	70%
177		1	100%	67%
178		4	100%	50%
179		3	100%	38%
Group Mean			**100%**	**56%**

1. Morbidity Incidence = (number of pigs with diarrhea / total pigs in litter) x 100.
2. Percent Mortality = (number of pigs that died from I. suis challenge / total number of pigs in litter) x 100.
3. Group A Gilt Vaccination: 3 intramuscular vaccinations at 5, 3 and 1 week pre-farrow with oocyst/sporozoite lysate.
4. Group B Gilt Vaccination: 200,000 I. suis oocysts orally at 6 weeks pre-farrow followed by intramuscular vaccinations with oocyst/sporozoite lysate at 5, 3 and 1 week pre-farrow.
5. Group C Gilt Vaccination: Non-vaccinated controls.
6. Group Mean: The statistical mean of each treatment group.

DISCUSSION

An Isospora suis sporozoite neutralizing antibody assay has been developed using a swine testicular cell line. The assay was used to demonstrate that I. suis antibodies produced in sera, milk and monoclonal antibody culture fluid inhibit the infection of I. suis sporozoites *in vitro*. The assay was also used to determine levels of I. suis neutralizing antibodies in post-vaccination milk samples and whether these titers correlated to passive protection observed in nursing pigs following an I. suis oocyst challenge.

EMNE media containing 2 to 5% bovine calf serum was used throughout the neutralization and adsorption steps of the assay because the serum assisted in both maintaining the viability of the sporozoites and supporting their attachment and penetration to ST cells. Fayer et al. (1984) described that after 120 minutes, excysted sporozoites failed to penetrate cultured cells due to a depletion of carbohydrate energy reserves. These results support the need for the calf serum to be present throughout the neutralization and adsorption steps in that components in the serum serve as an energy source for the sporozoites to become intracellular. Conversely, after adsorption during the final incubation step, it was imperative to use EMNE basal media without serum because the presence of serum aids in continued division of ST cells resulting in the absence of observable CPE.

Hyperimmune serum from guinea pigs and goats vaccinated with I. suis sporozoite preparations were evaluated using this sporozoite neutralizing antibody assay. These sera completely neutralized the sporozoites as demonstrated by lack of observable CPE indicating the penetration of I. suis sporozoites in ST cells was inhibited (Table 4). These results are similar to those reported for Eimeria tenella (Crane et al., 1986) and Plasmodium spp. (Ray et al., 1984; Guo et al., 1984).

The monoclonal antibody 2G3-H7, used in the evaluation of the *in vitro* sporozoite

neutralizing antibody assay, was specific for the apical complex of I. suis sporozoites as determined by indirect immunofluorescence. This monoclonal antibody, similar to that of E. acervulina reported by Sasai et al. (1996), inhibited the invasion of I. suis sporozoites *in vitro* (Table 5).

As indicated by the geometric mean titer of Group A in Experiment 1 (Table 6), the penetration of I. suis sporozoites into ST cells was inhibited by antibodies produced in milk samples from gilts inoculated three times orally with sporulated oocysts. These results are similar to those of Doyle et al. (1993) in which colostral antibodies from cows previously infected with Cryptosporidium parvum inhibited the penetration of homologous parasites in cultured cells.

The I. suis sporozoite neutralizing antibody assay was used to test milk samples from two animal experiments. To determine the effect of passive immunity from the gilts, the morbidity incidence and percent mortality for each litter of nursing pigs were calculated after the pigs were challenged orally with I. suis oocysts. To determine a correlation between protection and sporozoite neutralizing antibody titers, the morbidity and mortality data from each litter or group (Tables 7 and 9) were compared to the neutralizing antibody titers (Tables 6 and 8) in respective milk samples. There is no apparent correlation between the neutralizing antibody titers of individual gilts and their respective litter morbidity and mortality. Even when group geometric mean titer, morbidity incidence and percent mortality are compared, there is no correlation to neutralizing antibody titers.

Antibody titers in 3- and 5-day post-farrow milk samples were also determined using an I. suis oocyst/sporozoite ELISA (Tables 6 and 8). There was no definitive correlation between the sporozoite neutralizing antibody titers and the ELISA IgA or IgG antibody titers in the day of challenge milk samples in Experiments 1 or 2 (Tables 6 and 8). For example, Experiment 1 gilt #830 (Table 6) has a neutralizing antibody titer of 48 with an IgA titer of 32 and an IgG titer of 8, whereas in Experiment 2 gilt #180 (Table 8) has a

neutralizing antibody titer of only 3 but has an IgA titer of 512 and an IgG titer of 32. It is not surprising that there is a discrepancy between the sporozoite neutralizing antibody titers and the ELISA IgA and IgG titers since the ELISA is detecting antibodies against all oocyst and sporozoite antigens, while the neutralizing assay is detecting only the antibodies involved in inhibiting sporozoite attachment and penetration. Similar to the neutralizing titers, there is no correlation between ELISA antibody titers in milk samples and litter morbidity incidence and percent mortality in nursing pigs following challenge.

Even though the results of Experiments 1 and 2, indicate no correlation between neutralizing and ELISA antibody titers in day of challenge milk samples and protection against the clinical manifestations of an I. suis oocyst challenge, it is apparent that there may be other factors present aiding in the protection of the pigs suckling gilts that have been vaccinated with I. suis. First, it is likely the gilts had previously been exposed to a natural I. suis infection which may have resulted in the development of protective antibodies to either the endogenous stage or sexual stage of the parasite's life cycle. It is possible that the protective antibodies are against merozoites (type I and type II) because this stage is present for 2 to 5 days during the infection which may be long enough to elicit an antibody response. Conversely, sporozoites are present only for a few hours before becoming intracellular, which may not be long enough to elicit a good immune response. If merozoite neutralizing antibodies are present, then the infection would be allowed to proceed from excystation, through sporozoite attachment and penetration, to the endogenous development of the merozoites. After the merozoites are released into the intestinal lumen, maternal antibodies would neutralize and prevent merozoite infection. Second, the antibodies present in milk samples against the oocyst/sporozoite antigen, as determined by ELISA, are not neutralizing but may assist in the destruction of the parasite prior to infection of the gut epithelial cells. These antibodies may coat the intestinal mucosa of the pigs, thus inhibiting attachment and penetration of the sporozoites. Finally, the small

size of the treatment groups is a good possibility for the lack of correlation between neutralizing or ELISA antibody titers and protection against I. suis infection.

This I. suis sporozoite neutralizing antibody assay measures the level of neutralizing antibodies present in sera, milk and monoclonal antibody culture fluids and may benefit both pharmaceutical companies involved in the development of Isospora suis vaccines and diagnostic laboratories in monitoring outbreaks and collecting epidemiology information. However, as determined from vaccination-challenge animal experiments, the sporozoite neutralizing antibodies present are not necessarily protective and the absence of neutralizing antibodies does not necessarily imply lack of protection.

CONCLUSION

An *in vitro* Isospora suis sporozoite neutralizing antibody assay has been developed in swine testicular cells. The assay is performed in 96-well microtiter plates with confluent ST cell monolayers. I. suis sporozoites are excysted from purified oocysts, resuspended to a concentration of 4 to 8 x 10^5 sporozoites per milliliter in EMNE containing 2 to 5% bovine calf serum, and incubated with pre-diluted antibody samples for 60 minutes at 37°C. Neutralized sporozoites are inoculated at 0.05 ml per well, in duplicate, on ST cells and adsorbed at 37°C for 60 to 90 minutes. Following adsorption, ST cells are rinsed with EMNE and incubated at 37°C in a 3 to 5% CO_2 atmosphere. Intracellular sporozoites may be stained and read by indirect immunofluorescence after one hour incubation or stained with Diff-Quik (Baxter) differential stain after incubation for 6 to 8 days and read by cytopathic effect (CPE). This assay measures the level of neutralizing antibodies present in sera, milk and monoclonal antibody culture fluids by measuring both the level of intracellular sporozoites by immunofluorescence and the amount of CPE in ST cells. A monoclonal antibody against the apical complex of I. suis sporozoites inhibits infection of

sporozoites *in vitro* and hyperimmune sera produced in goats and guinea pigs inhibits sporozoite infection in ST cells. Milk samples from gilts vaccinated with I. suis contained neutralizing antibodies, however the titers did not correlate with protection observed in nursing pigs after challenge.

ADDENDUM 1 - Media Recipes

Phosphate-Buffered Saline, pH 7.2

8.6 mM Na_2HCO_3
3.4 mM NaH_2CO_3
168 mM NaCl
pH to 7.2 with 20% HCl or 10N NaOH

Sheather's Sugar Solution

667.6 g Sucrose
8.8 ml Formaldehyde
q.s. to 1 L with dH_2O

Bovine Calf Serum, Defined.
HyClone Laboratories, Inc., Logan, Utah. Cat. # A-2111-L.

EMNE - Earle's Salts Minimum Essential Medium with Non-Essential Amino Acids.
Irvine Scientific, Santa Ana, California. Cat. # 9478.

1L EMNE
2.0 mM L-glutamine
10 mM Sodium pyruvate
0.05-0.20 mg/ml gentamicin
2.2 mg/ml sodium bicarbonate
100 mM Hepes
pH to 7.2 to 7.4 with 10N NaOH or 1N HCl
if indicated 0.025 mg/ml trypsin
0.10 mg/ml DEAE Dextran

DMEM - Dulbecco's Modified Eagle's Medium, High Glucose.
HyClone Laboratories, Inc., Logan, Utah. Cat. # B-1006-BB.

1L DMEM
3.7 mg/ml sodium bicarbonate
0.20 mg/ml gentamicin
100 mM Hepes
pH to 7.2 to 7.4 with 10N NaOH or 1N HCl

LITERATURE CITED

Augustine, P. C. 1991. Monoclonal antibody produced against partially purified sporozoite antigen inhibits invasion of cells by sporozoites of avian *Eimeria* species. P.S.E.B.M. **198:** 606-611.

Bergeland, M. E. 1981. *Isospora suis* enteritis in piglets: diagnosis and epizootiology. Amer. Assn. Vet. Lab. Diag. 24th Annual Proceedings. 427-436.

Biester, H. E. and C. Murray. 1934. The occurrence of *Isospora suis* n. sp. in swine, a preliminary note. J. Am. Vet. Med. Assoc. **84:** 294.

Burkhardt, D. B. 1995. Ambico, Inc. Diagnostic Laboratory, Dallas Center, Iowa. Personal communication.

Crane, M. S., D. J. Norman, M. J. Gnozzio, A. C. Tate, M. Gammon, and P. K. Murray. 1986. *Eimeria tenella*: quantitative *in vitro* and *in vivo* studies on the effects of mouse polyclonal and monoclonal antibodies on sporozoites. Parasite Immun. **8:** 467-480.

Doyle, P. S., J. Crabb, and C. Petersen. 1993. Anti-*Cryptosporidium parvum* antibodies inhibit infectivity in vitro and in vivo. Infect. Immun. **61(10):** 4079-4084.

Driesen, S. J., P. G. Carland, and V. A. Fahy. 1993. Studies on preweaning piglet diarrhea. Aust. Vet. J. **70(7):** 259-262.

Dubremetz, J. F. 1993. Apical organelles (rhoptries, micronemes, dense granules) and host cell invasion by coccidia: what do we know now? VIth Int. Coccidiosis Conf. Guelph, Ontario, Canada.

Dudley, S. D. 1990. Veterinarians on call. National Hog Farmer. June 15: 72.

Eysker, M., G. A. Boerdam, W. Hollanders, and J. H. M. Verheijden. 1994. The prevalence of *Isospora suis* and *Strongyloides ransomi* in suckling piglets in the Netherlands. Vet. Quarterly. **16(4):** 203-205.

Fayer, R., H. R. Gamble, and J. V. Ernst. 1984. *Isospora suis:* Development in cultured cells with some cytological observations. Proc. Helminthol. Soc. Wash. **51(1):** 154-159.

Fayer, R. and M. C. Jenkins. 1992. Colostrum from cows immunized with *Eimeria acervulina* antigens reduces parasite development *in vivo* and *in vitro*. Poultry Science **71:** 1637-1645.

Fitzgerald, G. F., T. Barker, M. W. Welter, and C. J. Welter. 1988. Diarrhea in young pigs: comparing the incidence of the five most common infectious agents. Vet. Med. January: 80-86.

Grimwood, J. and J. E. Smith. 1996. *Toxoplasma gondii*: the role of parasite surface and secreted proteins in host cell invasion. International J. Parasit. **26 (2)**: 169-173.

Guo, S. C., W. E. Collins, C. C. Campbell, and W. Chin. 1984. *Plasmodium fragile*: inhibition of cultures by serum from rhesus monkeys immunized with homologous parasites. Exp Parasitol. **58(2)**: 156-162.

Harleman, J. H. and R. C. Meyer. 1985. Pathogenicity of *Isospora suis* in gnotobiotic and conventionalised piglets. Vet. Record **116**: 561-565.

Jarvinen, J. A. 1990. College of Veterinary Medicine, Iowa State University, Ames, Iowa. Personal communication.

Johnson, M. W., G. R. Fitzgerald, M. W. Welter, and C. J. Welter. 1992. The six most common pathogens responsible for diarrhea in newborn pigs. Vet. Med. April: 382-386.

Lindsay, D. S., B. P. Stuart, B. E. Wheat, and J. V. Ernst. 1980. Endogenous development of the swine coccidium, *Isospora suis* Biester 1934. J. Parasitol. **66(5)**: 771-779.

Lindsay, D. S., W. L. Current, and J. V. Ernst. 1982. Sporogony of *Isospora suis* Biester, 1934 of swine. J. Parasitol. **68(5)**: 861-865.

Lindsay, D. S., W. L. Current, J. V. Ernst, and B. P. Stuart. 1983a. Diagnosis of neonatal porcine coccidiosis caused by *Isospora suis*. Vet. Med./Small Anim. Clin. 89-94.

Lindsay, D. S., W. L. Current, and J. V. Ernst. 1983b. Motility of *Isospora suis* meronts. J. Parasitol. **69(4)**: 783-784.

Lindsay, D. S., W. L. Current, and J. V. Ernst. 1983c. Excystation of *Isospora suis* Biester, 1934 of swine. Z. Parasitenkunde **69**: 27-34.

Lindsay, D. S., J. V. Ernst, W. L. Current, B. P. Stuart and T. B. Stewart. 1984a. Prevalence of oocysts of *Isospora suis* and *Eimeria* spp from sows on farms with and without a history of neonatal coccidiosis. JAVMA. **185(4)**: 419-421.

Lindsay, D. S. 1984b. The biology of *Isospora suis* from swine. Ph. D. dissertation, Auburn Univ., Auburn, Alabama. 77 p.

Lindsay, D. S., W. L. Current, and J. R. Taylor. 1985. Effects of experimentally induced *Isospora suis* infection on morbidity, mortality, and weight gains in nursing pigs. Am. J. Vet. Res. **46(7)**: 1511-1512.

Lindsay, D. S. and B. L. Blagburn. 1987. Development of *Isospora suis* from pigs in primary porcine and bovine cell cultures. Vet. Parasit. **24**: 301-304.

Lindsay, D. S. 1989. Diagnosing and controlling *Isospora suis* in nursing pigs. Vet. Med. 443-448.

Lindsay, D. S. 1990. *Isospora*: infections of intestine: biology (Chapter 5). Long, P. L., ed. *Coccidiosis of Man and Domestic Animals*. CRC Press, 77-86.

Lindsay, D. S., B. L. Blagburn, and T. A. Powe. 1992a. Enteric coccidial infections and coccidiosis in swine. The Compendium/Food Animal **14(5)**: 698-701.

Lindsay, D. S., B. L. Blagburn, and B. P. Stuart. 1992b. Coccidia and other protozoa (Chapter 53). Leman, et. al., eds. *Diseases of Swine*. 7th ed. Ames, IA: Iowa State Univ. Press, 660-664.

Lindsay, D. S. and K. S. Todd, Jr. 1993. Coccidia of Mammals (Chapter 2). *Parasitic Protozoa*, vol. 4. Academic Press, Inc., 89-101.

Long, P. L. and M. E. Rose. 1972. Immunity to coccidiosis: effect of serum antibodies on cell invasion by sporozoites of *Eimeria in vitro*. Parasit. **65**: 437-445.

Marti, Jr., O. G. and O. M. Hale. 1986. Parasite transmission in confined hogs. Vet. Parasitol. **19**: 301-314.

Nilsson, O. and K. Martinsson. 1984. Neonatal coccidiosis in piglets. Svensk Veterinartidning. **36(7)**: 343-345.

Otten, A. 1995. Epidemiology and pathogenic significance of *Isospora suis* infection on ten piglet-rearing farms in North Rhine-Westphalia, Germany. Thesis, 110 p.

Ray, P., N. Sahoo, B. Singh, and F. A. Kironde. 1994. Serum antibody immunoglobulin G of mice convalescent from *Plasmodium yoelii* infection inhibits growth of *Plasmodium falciparum in vitro*: Blood stage antigens of *P. falciparum* involved in interspecies cross-reactive inhibition of parasite growth. Infect. Immun. **62(6)**: 2354-2361.

Robinson, Y. and M. Morin. 1982. Porcine neonatal coccidiosis in Quebec. Can. Vet. J. **23**: 212-216.

Robinson, Y., M. Morin, C. Girard, and R. Higgins. 1983. Experimental transmission of intestinal coccidiosis to piglets: Clinical, parasitological and pathological findings. Can. J. Comp. Med. **47**: 401-407.

Sangster, L. T., H. R. Seibold, and F. E. Mitchell. 1976. Coccidial infection in suckling pigs. Am. Assoc. Vet. Lab. Diagnosticians, 19th Annual Proc: 51-55.

Sangster, L. T., B. P. Stuart, D. J. Williams, and D. M. Bedell. 1978. Coccidiosis associated with scours in baby pigs. Vet. Med./Sm. Anim. Clin. 1317-1319.

Sasai, K., H. S. Lillehoj, H. Matsuda, and W. P. Wergin. 1996. Characterization of a chicken monoclonal antibody that recognizes the apical complex of *Eimeria acervulina* sporozoites and partially inhibits sporozoite invasion of CD8+ T lymphocytes in vitro. J. Parasitol. **82(1)**: 82-87.

Sayd, S. M. O. and U. Kawazoe. 1996. Prevalence of porcine neonatal isosporosis in Brazil. Vet. Parasitol. **67(3/4)**: 169-174.

South Dakota Animal Disease Research and Diagnostic Laboratory (ADRDL), Department of Veterinary Science, South Dakota State University, Brookings, South Dakota. Annual Report - Fiscal Years 1989-1995.

Stuart, B. P., D. S. Lindsay, and J. V. Ernst. 1978. Coccidiosis as a cause of scours in baby pigs. Proc. 2nd Int. Symp. Neonatal Diarrhea. 371-382.

Stuart, B. P. 1982. Coccidiosis in swine - a review. Meeting of the American Pork Congress. 13.

Stuart, B. P. and D. S. Lindsay. 1986. Coccidiosis in swine. Vet. Clin. North Am.: Food Anim. Pract. **2(2)**: 455-468.

Tubbs, R. C. 1986. A review of porcine neonatal coccidiosis. Food Anim. Pract. 899-903.

Tubbs, R. C. and M. A. Dekich. 1986. Neonatal coccidiosis in nine large Mississippi swine herds. Miss. Vet. J. **Fall:** 8-10.

Vetterling, J. M. 1966. Prevalence of coccidia in swine from 6 localities in the United States. Cornell Vet. **56:** 155-166.

Waddell, J. 1993. You can beat coccidiosis scours. Pork. January: 72.

Whitmire, W. M., J. E. Kyle, C. A. Speer, and D. E. Burgess. 1988. Inhibition of penetration of cultured cells by *Eimeria bovis* sporozoites by monoclonal immunoglobulin G antibodies against the parasite surface protein P20. Infect. Immun. **56(10)**: 2538-2543.